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Analysis of the HIV-1 *nef* Gene in Five Intravenous Drug Users With Long-Term Nonprogressive HIV-1 Infection in Italy

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Great variability in the course of human immunodeficiency virus type 1 (HIV-1) infection results from a complex interplay between host and virus factors. Some of the patients with prolonged nonprogressive infection have been reported to harbor virus variants with gross deletions in the accessory *nef* gene that has been implicated in in vivo pathogenicity in simian and mouse models. To investigate the role of *nef*-deleted HIV-1 in long-term nonprogressor (LTNP) drug addicts in Italy the *nef* sequence from proviral DNA was analyzed from five LTNPs and five rapid progressor controls. Only small (2–12 amino acids) in-frame deletions and insertions were detected in the N-terminal polymorphic and variable regions obtained from three LTNPs and one rapid progressor. There was no evidence of premature termination of the Nef protein and all of the identified functional motifs were well conserved in both groups. Phylogenetic analysis showed interdigitation of *nef* sequences obtained from LTNPs and rapid progressors. The *nef* sequence of one LTNP, however, diverged significantly from those of the other patients. Availability of two additional blood DNA samples obtained previously from this subject allowed to detect evolution of *nef* at 14–17 years of HIV-1 infection, including progressive deletions. Although alterations of *nef* may be relatively frequent and continue to evolve in LTNPs, this study of a small number of patients does not indicate that gross deletions or loss of functional motifs play a major role in delaying or halting disease progression in infected drug abusers in Italy. ***J. Med. Virol. 60:294–299, 2000.***

KEY WORDS: long-term nonprogressors; rapid progressors; sequence analysis

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) establishes a chronic infection characterized by a significantly variable course depending on a complex interaction between host and virus factors [Fauci, 1996; Antia and Halloran, 1996; Cohen et al., 1997]. Infection with HIV-1 variants with attenuated replicative capability may have contributed to the beneficial course of disease in a minor group of subjects termed long-term nonprogressors [LTNPs] who have remained healthy and immunocompetent for more than a decade in the absence of antiretroviral treatment. [Barker et al., 1998].

The viral *nef* accessory gene has been associated with pathogenesis in vivo in the simian immunodeficiency virus (SIV) [Kestler et al., 1991], SCID-human [Jamieson et al., 1994] and transgenic mouse [Hanna et al., 1998] models and has been found to be defective in several human LTNPs [Huang et al. 1995; Deacon et al., 1995; Kirchhoff et al., 1995; Mariani et al., 1996, Michael et al., 1995; Premkumar et al., 1996; Salvi et al., 1998]. A number of functions have been consistently attributed in vitro to wild type *nef* alleles including down-regulation of the cell surface CD4 receptor and MHC-I molecules [Mangasarian et al., 1999; Greenberg et al., 1998; Hua et al., 1997], modulation of cellular activation and signal transduction pathways [Sawai et al., 1994; Baur et al., 1997], and enhancement of viral infectivity [Guatelli, 1997]. All of these functions are strictly dependent on the subcellular localization of Nef and its transient association with the plasma membrane via *N*-myristoylation at Gly-2 and targeting by an N-terminal cluster of basic amino acids [Welker et al., 1998]. Once incorporated into the virion, Nef is cleaved by the viral protease between Trp-57 and Leu-58 to yield the C-terminal core domain [Pandori et al., 1998]. This contains repeated Pro-X-X-Pro domains involved in mediating efficient proviral DNA synthesis [Wiskerchen and Cheng-Mayer, 1996; Aldrovandi et al., 1998]. Additional critical regions have been identi-

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fied in the core domain including a pair of basic residues involved in cell surface CD4 downmodulation [Wiskerchen and Cheng-Mayer, 1996] and a dileucine motif targeting membrane proteins to clathrin-coated vesicles. [Greenberg et al., 1998]. Whereas the C-terminal Nef core is conserved in SIV and HIV-1 isolates the N-terminal domain is more variable and contains a highly polymorphic region [codons 8–15] that serves as a nuclear localization signal essential for the transcriptional effects of Nef [Murti et al., 1993].

Although at least some of the in vitro functions of Nef are expected to influence HIV-1 replication in vivo, their relative contribution toward AIDS pathogenesis have not yet been defined. Most data on nef structure in LTNPs have been obtained from patients infected by blood product transfusion and sexual contacts [Deacon et al., 1995; Huang et al. 1995; Kirchhoff et al., 1995; Michael et al., 1995; Mariani et al., 1996, Premkumar et al., 1996; Salvi et al., 1998; Vallejo et al., 1999]. To contribute additional data on the possible association between Nef variants and the rate of disease progression, we have directly isolated and analyzed *nef* alleles from clinical specimens of LTNP and rapid progressor drug addicts by nested PCR and DNA population sequencing.

MATERIALS AND METHODS

Five intravenous drug abusers were studied as LTNPs (L1 to L5) on the basis of asymptomatic HIV-1 infection for more than 13 years (range 14–17) in the absence of antiretroviral therapy and with $CD4^+$ T cell counts remaining above 500/mm^3 (range 630–1173 at the time of sampling). A single sample was available for each subject except for Patient L1 for whom three samples obtained at 15, 15.5 and 17 years of infection were examined. As control subjects, five rapid progressor drug addicts (R1 to R5) were also studied on the basis of clinical progression to CDC stage B3 or C3 within four years since documented seroconversion.

HIV-1 DNA burden in peripheral blood mononuclear cells (PBMCs) and plasma HIV-1 RNA load were quantitated by competitive PCR and RT-PCR methods, respectively, as described [Zazzi et al., 1996; Zazzi et al., 1999]. The Nef-encoding proviral DNA region was amplified by nested PCR with outer primers NEF-1 (5′ CAGTAGCTGAGGGGACAGATAG 3′, position 8693–8712 in the HIV-1 SF2 strain, GenBank accession number KO2007) and NEF-2 (5′ CCACTCCCCAGTCCCGCCC 3′; position 9481–9500) and inner primers NEF-3 (5′ CATACCTAGAAGAATAAGACAGGG 3′; 8757–8380) and NEF-4 (5′ CCCAGCGGAAAGTCCCTTGTAG 3′; position 9436–9457) using 1 μg of PBMC DNA as starting material. Crude PCR products were then used directly as templates for a cycle sequencing reaction directed by the infrared-labeled sense primer IR-8 (5′ CCTAGAAGAATAAGACAGGGCTTG 3′, position 8761–8785) and antisense primer IR-9 (5′ AGCGGAAAGTCCCTTGTAGCAA 3′, position 9433–9455). Termination products were electrophoresed and analyzed in a Model 4000L Licor DNA Sequencer. Details of the entire sequencing procedure have been reported [Zazzi et al., 1998].

To investigate length variation in *nef* gene, PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis. Nucleotide and amino acid sequences were aligned by CLUSTALW program [Higgins et al., 1992] to the HIV-1 subtype B *nef* consensus available at the Los Alamos HIV Sequence Database [Korber et al., 1997]. Interpatient distances were computed by DNADIST of Phylip version 3.57 package [Felsenstein, 1989] according to the Kimura two-parameter model. Specific changes in putative functional motifs within *nef* sequence were investigated by PROSITE program [Hofmann et al., 1999]. Nucleotide and amino acid sequences were subjected to phylogenetic analyses with the neighbor-joining method as implemented by DRAWTREE software contained in the same Phylip package. Sequences have been deposited to GenBank under accession numbers AF147728 through AF147737.

RESULTS AND DISCUSSION

All of the patients studied had detectable HIV-1 DNA in PBMCs with higher levels in the rapid progressors than in the LTNPs (median 12,897 vs. 907 copies/10^6 $CD4^+$ T cells, respectively; $P = 0.01$). Relatively low levels of plasma HIV-1 RNA were measured in four of the five LTNP (median 5,585 copies/ml, range 1,260–10,733) although Patient L5 had undetectable plasma viremia (<400 copies/ml). Plasma HIV-1 RNA levels in the five rapid progressors were controlled partially by highly active antiretroviral therapy but had peaked above 100,000 copies/ml before treatment (data not shown).

Gel electrophoresis of amplified *nef* DNA revealed no major length variation in any patient; however, when the deduced amino acid (aa) sequences were aligned several in-frame deletions and insertions of 1–10 residues were detected outside the C-terminal core region (Fig. 1). A 2-aa or 4-aa deletion was present in the highly polymorphic region in three of the five LTNPs (L1, L2, L3) but in none of the five rapid progressors. Deletions in this same region have been documented previously in some LTNPs and occasionally in rapid progressors [Huang et al., 1995; Michael et al., 1995;]. A 4-aa insertion at codon 23 in the variable region was found in Patients L2 and R1. A longer (10 aa) insertion at the same codon was detected in Patient L1. Different insertion at this position have been reported in infected patients independently of the rate of disease progression [Huang et al., 1995; Michael et al., 1995; Blaak et al., 1998; Vallejo et al., 1999]. Nef from Patient L1 only additionally had a 2-aa deletion nine codons upstream the cleavage site. No such deletion has been reported in previous LTNP studies. The only length variation located in the C-terminal core region was a 1-aa deletion in the acidic domain immediately preceding the first Pro-X-X-Pro motif in all patients except for L1, R3 and R4. This deletion seems to be a common feature in HIV-

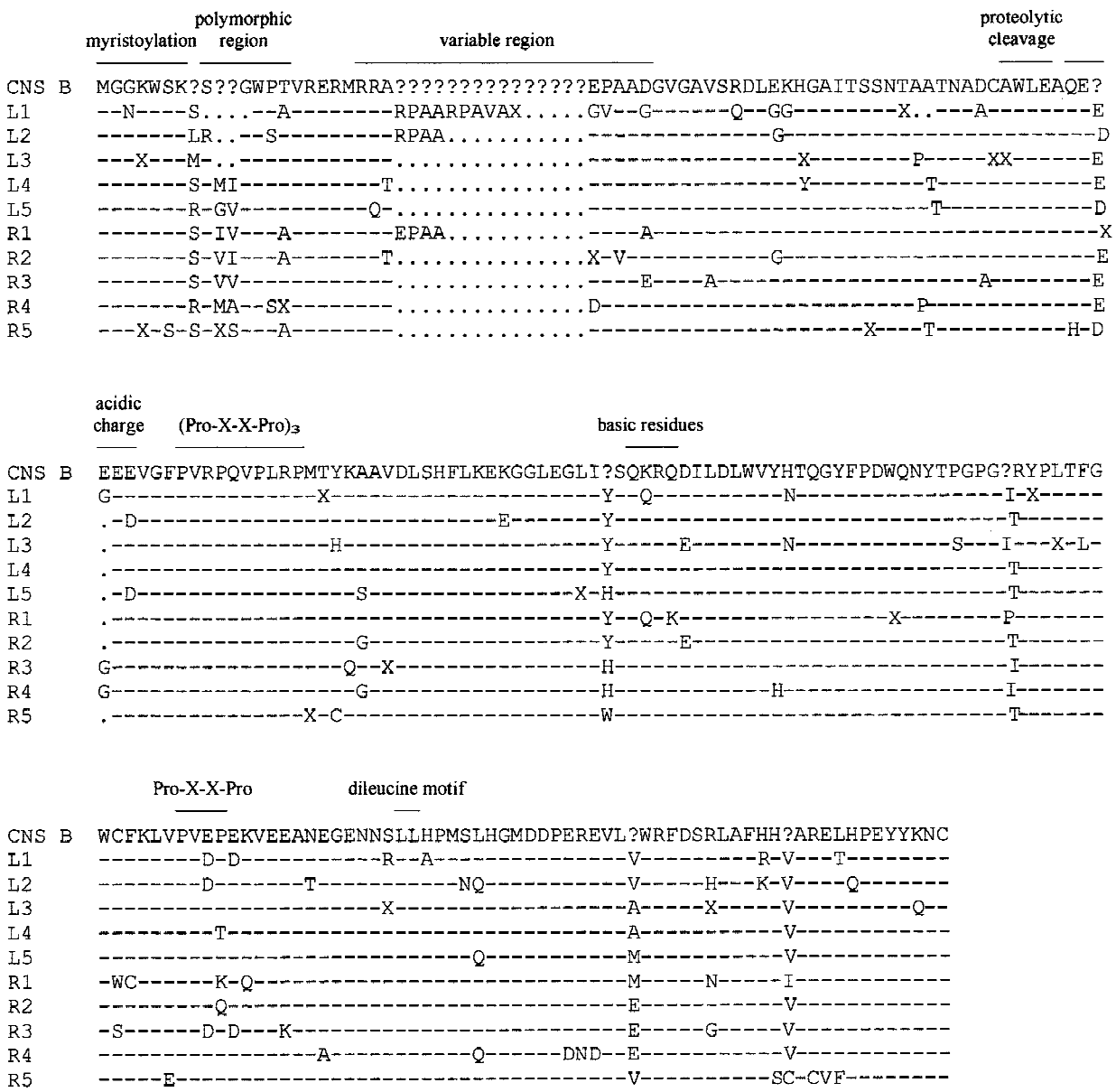

Fig. 1. Alignment of the deduced amino acid Nef sequences obtained from five long-term nonprogressors (L1–L5) and five rapid progressors (R1–R5). Sequences were aligned to HIV-1 subtype B consensus Nef. Defined regions and functional domains are indicated above the consensus sequence. Dashes indicate identity to the consensus, dots indicate gaps introduced to maximize the alignment. An "X" indicates a mixture of two different amino acids deduced from population sequencing.

1-infected individuals [Huang et al., 1995; Blaak et al., 1998; Vallejo et al., 1999].

Archival samples obtained 18 and 24 months before were also available for the patient (L1) who harbored unique *nef* variations in the population analyzed. Alignment of all the predicted aa sequences from this patient (Fig. 2) showed that the 4-aa deletion in the highly polymorphic region occurred between the first and second time point considered. Interestingly, at the same time a duplicated Arg-Ala motif was apparently lost from a 12-aa insertion at codon 23 yielding the 10-aa insertion observed thereafter. By contrast, the 2-aa deletion near the proteolytic cleavage site was present in all of the three sequences.

None of the aa substitutions detected in our LTNPs and rapid progressors caused premature termination of the Nef protein. All of the critical regions were well conserved in the whole population including the initiator methionine, the *N*-myristoylation site, the cleavage site, the Pro-X-X-Pro domains and the dileucine motif. A possible internal initiator codon (Met-20) [Kaminchik et al., 1991] was also conserved in both groups. A protein kinase C phosphorylation site at Thr-15 [Kaminchik et al., 1991] was altered to Ala in Patient L1 and in three rapid progressors. Abolishment of this phosphorylation site has been previously noted in HIV-1-infected patients, including LTNPs [Huang et al., 1995]. The presence of Ala at this position abrogates

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CNS B     MGGKWSK?S??GWPTVRERMRRA????????????????EPAADGVGAVSRDLEKHGAITSSNTAATNADCAWLEAQE?

14 yrs    MGNKWSKSKAGGWPAVRERMRRARARPAARPAVAE...GVVAXGVGAVSQDLGKHGAITSSNL..NNAACAWVEAQEE
14.5 yrs  MGNKXSKS....WPAVRERMRRA..RPAARPAVAE...GVXAEGVGAVSQDLGKHGAITSSNL..NNAACAWVEAQEE
17 yrs    MGNKWSKS....WPAVRERMRRA..RPAARPAVAX...GVAAXGVGAVSQDLGXHGAITSSNX..NNAACAWVEAQEX
          **** ***    ***********  *********    ** * ********** ********   ************


CNS B     EEEVGFPVRPQVPLRPMTYKAAVDLSHFLKEKGGLEGLI?SQKRQDILDLWVYHTQGYFPDWQNYTPGPG?RYPLTFG

14 yrs    GEEVGFPVRPQVPLRPMTYKAALDLSHFLKEKGGLEGLIYSQQRQDILDLWVYNTQGFFPDWQNYTPGPGIRHPLTFG
14.5 yrs  GEEVGFPVRPQVPLRPMTYKAALDLSHFLKEKGGLEGLIYSQQRQDILDLWVYNTQGFFPDWQNYTPGPGIRXPLTFG
17 yrs    GEEXGFPVRPQVPLRPMXYKAALDLSHFLKEKGGLEGLVYSQQRQDILDLWVYNTQGFFPDWQNYTPGPGXRXPLTFG
          *** ************* ********************.****************************** * *****


CNS B     WCFKLVPVEPEKVEEANEGENNSLLHPMSLHGMDDPEREVL?WRFDSRLAFHH?ARELHPEYYKNC

14 yrs    WCLKLVPVEPDRVEEANEGENNSLLPPMSLHGMDDPEKEVLXWRFDSRLAFHNVAKETHPEFYKER
14.6 yrs  WCLKLVPVEPDKVEEANEGENNRLLAPMSLHGMDDPEKEVLVWRFDSRLAFHHVAKETHPEFYKDC
17 yrs    WCFKLVPVDPDKVEEANEGENNRLLAPMSLHGMDDPEKEVLVWRFDSRLAFRHVARETHPEFYKDC
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Fig. 2. Alignment of the deduced amino acid Nef sequences obtained from Patient L1 at 14 (L1 a), 14.5 (L1 b) and 17 (L1 c) years since documented seroconversion. Asterisks and dots indicate identity and functional conservation, respectively, among the L1 sequences. The reference HIV-1 subtype B consensus Nef is shown above the sequences from Patient L1. An "X" indicates a mixture of two different amino acids deduced from population sequencing.

transcriptional effects of Nef without affecting the ability to downregulate cell surface CD4 expression [Bandres et al., 1994]. A potential Asn-linked glycosylation site upstream the dileucine motif was altered in Patient L1 only. This substitution occurred between the first and second time points considered for this patient [Fig. 2]. This glycosylation site has been reported to be abrogated in several LTNPs and rapid progressors [Huang et al., 1995; Vallejo et al., 1999]. Finally, we did not observe the recently reported association between an Ile→Val substitution at codon 11 and the LTNP status [Visco-Comandini et al., 1998].

The mean interpatient distance among LTNP *nef* sequences was similar to that among rapid progressor *nef* sequences (0.100 and 0.092, respectively). The mean interpatient distance between LTNPs and rapid progressors was also comparable to the values obtained for within group analysis (0.980). Indeed, phylogenetic analysis revealed interdigitation of LTNP and rapid progressor *nef* sequences, indicating no apparent clustering with the disease progression rate (Fig. 3), similar to a previous report [Huang et al., 1995]. The mean distance between Patient L1 and the others, however, was significantly larger than the mean distance among the other patients (mean ± SD, 0.151 ± 0.031 vs. 0.084 ± 0.019; $P < 0.001$), confirming that the *nef* sequence from this LTNP had unique features in the group studied.

In summary, although this study of a small number of subjects does not allow general conclusions about the frequency of extended *nef* deletions in long-term nonprogressive HIV-1 infection the results obtained suggest that specific *nef* alterations are not a frequent feature of LTNP drug addicts. Whereas gross *nef* deletions have been documented anecdotally in LTNPs [Kirchhoff et al., 1995; Mariani et al., 1996; Salvi et al., 1998], several small comparative studies have failed to detect any *nef* sequence pattern discriminating LTNPs from rapid progressors in subjects who acquired HIV-1 infection mainly by transfusion or the sexual route [Huang et al., 1995, Michael et al., 1995; Blaak et al., 1998; Vallejo et al., 1999]. In addition, disease progression has been reported recently in a LTNP harboring a grossly *nef*-deleted virus [Greenough et al., 1999].

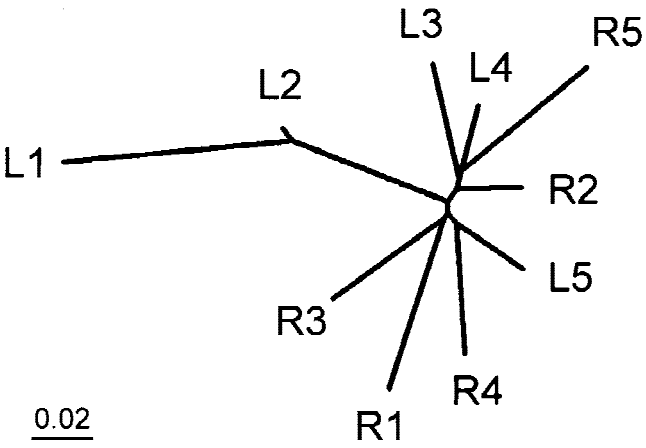

Fig. 3. Phylogenetic relationship of *nef* nucleotide sequences from five long-term nonprogressors (L1–L5) and five rapid progressors (R1–R5). The phylogenetic tree was constructed by the neighbor-joining method (see Materials and Methods).

It is interesting to note that *nef* sequences were quite homogeneous within each of the LTNPs and rapid progressors studied. Indeed, we used direct population sequencing of PCR products generated from 1 μg of PBMC DNA representing 9–265 HIV-1 DNA copies in

the group studied and detected a unique sequence with a very low number of ambiguous base callings and no length polymorphism. Even the shift from the first to the second *nef* sequence obtained from Patient L1 involving two separate deletions seemed to be complete within a period of six months. Progressive loss of *nef* portions has been suggested recently to have occurred also in the Sydney blood bank LTNP cohort infected originally with a *nef*-deleted attenuated virus [Greenway et al., 1998], suggesting that continuous evolution of *nef* occurs in the presence of and possibly contributes to nonprogressive infection. Present availability of simple molecular techniques makes it advisable to screen *nef* and other HIV-1 accessory genes in larger numbers of infected individuals with different progression rates to clarify which viral factors play a crucial role in the variable course of infection. Such studies should be properly supplemented with analysis of host factors (e.g., HIV-1 coreceptor modifications, HIV-1-specific cytotoxic T lymphocyte responses) [Cohen et al., 1997; Berger et al., 1999] expected to be involved in modulating disease progression.

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